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Substance P accelerates wound repair by promoting neovascularization and preventing inflammation in an ischemia mouse model

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ABSTRACT

Aims: Arterial insufficiency ulcers are frequent complications of peripheral artery disease and infection or long-term neglect of the ulcer can eventually lead to amputation of the affected body part. An ischemic environment, caused by interrupted blood flow, affects the supply of nutrients and elongates the inflammation period, inducing tissue degeneration. Thus, the modulation of neovascularization and inflammation could be an ideal therapeutic strategy for ischemic wound healing. This study aimed to elucidate whether systemically administered substance P (SP) could promote ischemic wound repair in mice by restoring blood perfusion and suppressing inflammation.

Main methods: The effects of SP were assessed by analyzing wound size, blood flow, epidermal and dermal layer regeneration, vessel formation, and the inflammatory cytokine profiles in a hind-limb ischemia non-clinical mouse model.

Key findings: SP-treated mice exhibited dramatically rapid wound healing and restoration of blood flow within the ischemic zone, compared with saline-treated mice. Notably, SP-treated mice showed enhanced pericyte-covered vasculature compared to saline-treated mice. Moreover, anti-inflammatory effects were detected in mice in the SP-treated group, including suppression of inflammation-mediated spleen enlargement, reduction of tumor necrosis factor-alpha, and promotion of circulatory interleukin-10 levels.

Significance: These results suggest that SP could be a possible therapeutic candidate for patients with peripheral artery disease, including those with ischemic ulcers.

1. Introduction

Peripheral artery disease (PAD) is a frequently occurring degenerative vascular disease that leads to inadequate blood flow [1]. Interruption in blood flow decreases blood supply to tissues, hindering the delivery of adequate nutrients and oxygen. If there is prolonged deficiency in blood supply, arterial insufficiency ulcers, known as ischemic ulcers, develop, accompanied by inflammation. Unless tissue perfusion is corrected, the damage may lead to infection and subsequent amputation [2].

The optimal therapeutic target for PAD treatment is recovery of blood perfusion via formation of new blood vessels in ischemic tissue [3] so that cellular response within the ischemic zone can occur normally with growth factor, cytokine, and immune cell infiltration [4]. In tissue ischemia, blood perfusion occurs through angiogenesis and arteriogenesis [5]. While endothelial cells are the primary target of angiogenic factors, arteriogenic factors target perivascular cells, including pericytes and vascular smooth muscle cells. Endothelial and perivascular cells can generate functional collateral networks. Thus, a combination of angiogenic and arteriogenic factors is essential for vascular remodeling and maintenance of the integrity of the vasculature. Platelet-derived growth factor [6], angiopoietin [7], vascular endothelial growth factor (VEGF), transforming growth factor-beta (TGF-β) [8,9], fibroblast growth factors [10], and hepatocyte growth factor play

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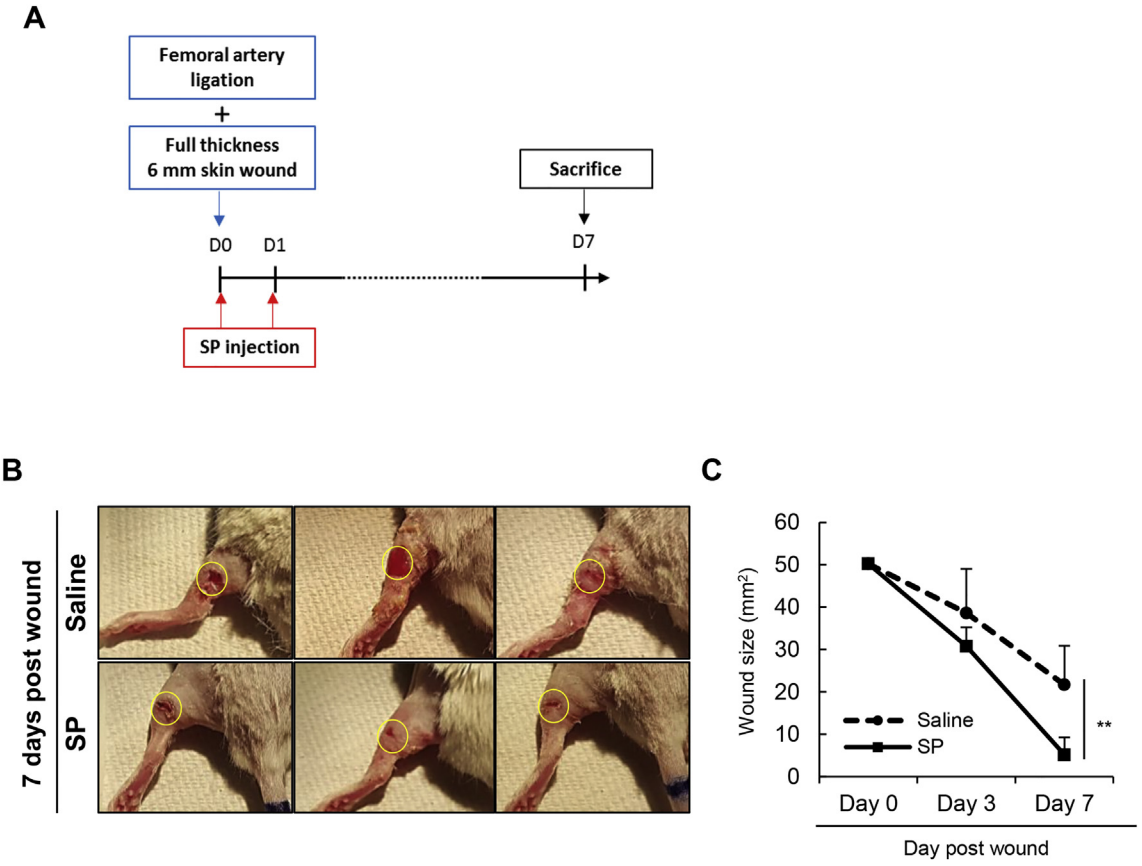

Fig. 1. SP accelerates wound closure under ischemia.
(A) A schema for the animal disease model with SP treatment. (B) A gross view of the wounds at 7 days after they were created. The yellow-dotted circles indicate the wounded area. (C) Wound size was measured and quantitatively evaluated for 7 days. Data are shown as mean values ± standard deviations. (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

important roles in angiogenesis and arteriogenesis [11].

Recently, single gene/protein-based and cell-based strategies, as well as angiogenic growth factor therapy, have been implemented for ischemic disease treatment [12–14]. However, to date, combinations of angiogenic and arteriogenic factors have been demonstrated to synergistically improve the functional outcomes of ischemic tissues [15]. Thus, multiple controls for angiogenic and arteriogenic potentials should be considered.

Bone marrow mesenchymal stem cells (BMSCs) can differentiate into perivascular cells [16]. Perivascular cells play a significant role in promoting wound healing as components of functional mature vessels, improving perfusion in ischemic tissues to elevate aerobic energy metabolism in muscles. Furthermore, BMSCs not only secret VEGF and platelet-derived growth factor, which are essential to angiogenesis, but also suppress inflammatory responses. Thus, mobilizing BMSCs to circulation is effective in repairing ischemic ulcers by promoting stem cell participation and facilitating angiogenesis- and arteriogenesis-related factors.

Substance P (SP) modulates the proliferation and migration of vascular endothelial cells [17–20]. It protects the vasculature from inflammation-induced cell death [21], improves the angiogenesis-related paracrine potential [22], and can mobilize BMSCs to circulation to engage in tissue repair, accompanied by angiogenesis and VEGF production [23–25]. Repeated application of SP accelerates wound closure in non-diabetics and diabetics [26,27]. During wound healing, SP shows anti-inflammatory effects by increasing M2 macrophage and regulatory T cell numbers and interleukin-10 (IL-10) levels and decreasing tumor necrosis factor-alpha (TNF-α) levels [28–30,39].

Because PAD is reportedly associated with inflammation and dysfunction of the vasculature, [31] it was thought that SP might exert a therapeutic effect in ischemic injury by modulating vascular activity and immune responses.

Therefore, this study aimed to elucidate whether systemically administered SP could promote the repair of ischemic wounds, based on its effects in terms of cutaneous healing, cytokine profile, and blood flow.

2. Materials and methods

2.1. Materials

SP was purchased from Sigma-Aldrich (St. Louis, MO). Tegaderm film was purchased from 3 M Health Care (St. Paul, MN) and Mepitel dressings were purchased from Mölnlycke Health Care (Gothenburg, Sweden). A 6-mm biopsy dermal punch was purchased from Kai Medical (Tokyo, Japan). Mouse TNF-α, IL-10, TGF-β and VEGF enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D (Minneapolis, MN).

2.2. Experimental animals

Six-week-old Balb/c mice (23 g, male) were purchased from Daehan Bio Link (Seoul, Korea). All of the animals were maintained under a regular light/dark illumination cycle in an animal holding room and allowed to acclimatize for 7 days before the experiments. All animals received a standard chow diet. This study was approved by the Ethical Committees for Experimental Animals of Kyung Hee University with the approval number of KHMC-IACUC 2017-005.

2.3. Surgery for hind-limb ischemia and skin wounds

All mice were anesthetized intraoperatively using intraperitoneal injections of ketamine (100 mg/kg, Yuhan, Seoul, Korea) and Rompun (1.2 mg/kg, Bayer Healthcare, Kyunggi-do, Korea). To induce hind-limb ischemia, the femoral artery was ligated and its branches were resected without disturbing the neurons. After surgery, a full-thickness wound within the ischemic area was induced using a 6-mm biopsy punch. SP was intravenously injected twice per week according to the schedule shown in Fig. 1A. The wound was covered with Mepitel and Tegaderm. The dressing was changed once every 3 days. The mice were randomly divided into two groups: (1) the hind-limb ischemia ulcer with a vehicle (saline) group and (2) the hind-limb ischemia ulcer with SP group.

Postoperatively, the blood flow of the hind-limb was monitored using a laser Doppler perfusion system (Moor Instruments, Devon, UK). Blood flow from the knee joint to the toe region was analyzed to calculate the perfusion rate.

Wound healing was monitored for 7 days and the wound size was quantified on days 3 and 7. To further evaluate the effect of SP on re-epithelialization and collagen deposition, the wound was histologically evaluated 7 days after it was induced. To quantitatively analyze the formation of vessels, the total vasculature and coverage of pericytes in the skin and muscle layer within the wound site were assessed, respectively.

2.4. Administration of SP

SP was diluted in saline (JW Pharmaceuticals, Seoul, Korea) immediately before use and administered intravenously twice a week at a dose of 5 nmol/kg. Saline was used as the vehicle.

2.5. Histological analysis

The mice were sacrificed 7 days after wounding. The skin, muscle, and spleen were isolated and fixed in 3.7% paraformaldehyde (PFA, Sigma-Aldrich) for 1 day. Samples were processed with a TP1020 tissue processor (Leica Biosystems, Wetzlar, Germany) to make paraffin blocks, and 4.0-μm-thick sections were prepared. For hematoxylin and eosin staining, sections of the samples embedded in paraffin were hydrated in alcohol for 2 min. After the sections were washed in tap water, they were stained with hematoxylin (Sigma-Aldrich) for 2 min and washed in tap water again. Eosin Y (Sigma-Aldrich) was used to stain the cytoplasm and the sections were then washed with tap water. Trichrome staining was performed using the NovaUltraTM Masson Trichrome Stain Kit (IHC World, Woodstock, MD).

Immunohistochemical staining was performed following the VECTASTAIN ABC Kit protocol (Vector Laboratories, Burlingame, CA). Briefly, samples were treated with 0.5% H_2O_2 to block the activity of endogenous hydrogen peroxidase (HRP) and then permeabilized with 0.3% Triton-X 100. Nonspecific binding was blocked by incubating the samples in 2% normal horse serum for 1 h at room temperature (RT). The sections were incubated with primary antibodies against alpha-smooth muscle actin (α-SMA, 1: 100; Abcam), CD31 (1:100), or CD206 (1:200). The samples were then washed in phosphate-buffered saline three times and incubated with a biotin-conjugated secondary antibody for 1 h at RT. After washing the sample in phosphate-buffered saline, substrate solution with HRP or alkaline phosphatase was added, and the mixture was incubated for 1 h at RT. To visualize the reactive area in the tissue, Nova Red (Vector Laboratories) or BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium, Vector Laboratories) was added to the samples, after which the nuclei were counterstained with Fast Red for 10 min. Then, the samples were mounted.

2.6. Quantification of the wound area

To quantitatively analyze the histological outcomes, the wound area was measured using a modified version of previously described methods [32]. Epithelial coverage was evaluated by analyzing the migration of epithelial cells into the wound site. Formation of granulation tissue was estimated based on the infiltration of fibroblasts and endothelial cells, as well as the invasion of immune cells. The thickness of the granulation tissue was assessed with respect to the underlying muscle fascia. All quantitative analyses were performed by assessing more than three adjacent fields on tissue slides.

2.7. ELISA

The concentrations of TNF-α, IL-10, TGF-β and VEGF in serum samples were measured using an ELISA kit according to the manufacturer's instructions. Briefly, all reagents, standard dilutions, and samples were prepared as directed, and then 100 and 50 μl of calibrator diluent were added to nonspecific binding and zero-standard wells, respectively. To the remaining wells, 50 μl of the standard, control, or sample solution was added. Next, 50 μl of a secondary antibody solution was added to each well, followed 2 h later by 100 μl of a conjugate solution. Finally, 100 μl of substrate solution was added to each well. Once the color of the solution changed to blue, the reaction process was stopped and the optical density was measured, with the wavelength correction set to 450 nm, using an EMax Endpoint ELISA Microplate Reader (Molecular Devices, Sunnyvale, CA).

2.8. Fluorescence-activated cell sorting (FACS) analysis

To quantify the $CD29^+CD105^+CD45^-$MSC in the PBMCs, 2×10^7 mononuclear cells were incubated with a fluorescein-5-isothiocyanate (FITC)-conjugated anti-CD29 antibody, allophycocyanin (APC)-conjugated anti-CD105 antibody, and phycoerythrin (PE)-conjugated anti-CD45 antibody (all from Miltenyi, Bergisch Gladbach, Germany). To detect M2-type monocytes, 2×10^7 PBMCs with an FITC-conjugated anti-CD11b antibody (Miltenyi, Bergisch Gladbach, Germany) and Cy 5.5-conjugated anti-CD206 antibody (Biolegend, San Diego, CA). The BMSC fractions or M2 monocytes in circulation were analyzed using a FACSCalibur Flow Cytometer using the CellQuest software (Becton Dickinson, San Jose, CA).

2.9. Statistics

Data are presented as the mean ± standard deviation of three independent experiments. p-Values < 0.05 were considered statistically significant. Statistical analysis was performed using an unpaired two-tailed Student's *t*-test.

3. Results

3.1. Under ischemic conditions, SP promotes wound healing accompanied by recovery of blood flow

A full-thickness wound was created in an ischemic zone and SP was injected once a day for 2 days. Monitoring for wound size was performed for 7 days (Fig. 1A). As shown in Fig. 1B–C, in the SP-injected group, wound closure was accelerated and the wounds were almost healed at 7 days after creation, while mice in the saline-treated group showed delayed healing.

In order to determine epithelial migration and recovery of granulation tissue, histological analysis of the wounded region was performed. The wound site of mice in the saline-treated group was rarely covered with epithelial cells, whereas that of mice in the SP-treated group displayed an almost fully covered epithelial layer (Fig. 2A–B, saline: 37.27 ± 18.08, SP: 94.14 ± 9.87, saline vs SP: $p < 0.001$). Furthermore, an analysis of the formation of granulation tissue revealed that mice in the SP-treated group had a greater amount of recovered granulation tissue with collagen deposition and neovascularization than

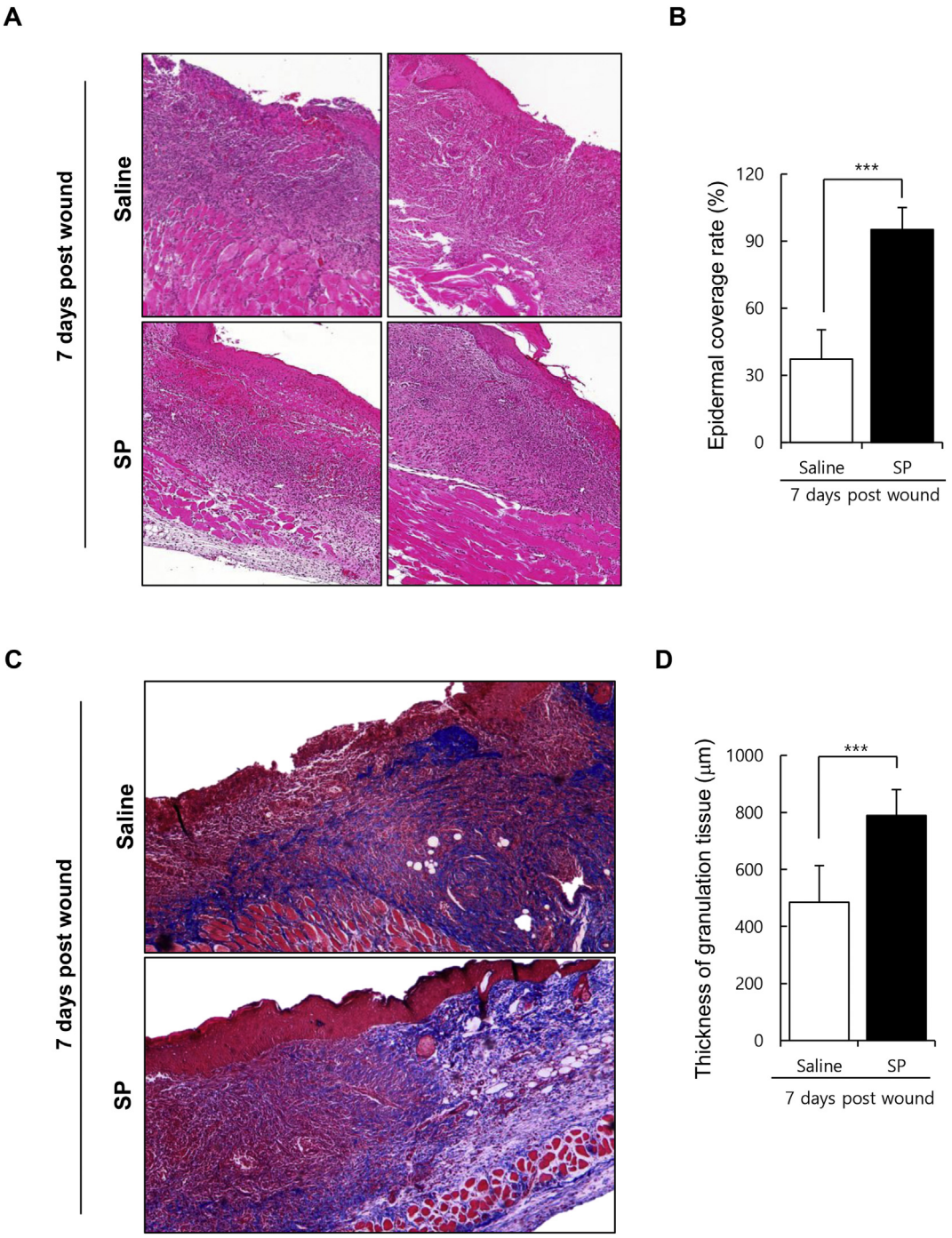

Fig. 2. SP promotes ischemic wound healing by accelerating the recovery of the epidermal and dermal layers.
(A) Hematoxylin and eosin staining of the wounds was conducted at day 7 after they were created. (B) The epidermal coverage rate was calculated and quantitatively evaluated using the ImageJ program. (C) Masson's trichrome staining of the wounds was performed on day 7 after they were created. (D) Thickness of the granulation tissue was measured and quantitatively evaluated using the ImageJ program. Data are shown as mean values ± standard deviations. n = 8 per group. (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

did those in the saline-treated group (Fig. 2C–D saline: 484.89 ± 128.69, SP: 789.18 ± 90.36, saline vs SP: $p < 0.001$).

Ischemic wound healing is mostly impaired due to insufficient supply of nutrients and growth factors into the injured site. Thus, the accelerated wound healing by SP treatment implies sufficient supply of the reparative factors through the blood stream. To examine whether SP-mediated wound healing is accompanied by restoration of blood flow, blood flow in limbs was comparatively analyzed between the groups using Laser Doppler. A distinct difference in the blood flow between both groups was rarely detected early; however, beginning on day 4, mice in the SP-treated group showed rapid recovery of blood reperfusion compared with those in the saline-treated group (Fig. 3A–B).

These data suggest that systemically administered SP can promote wound healing under the ischemic condition, with restoration of blood flow.

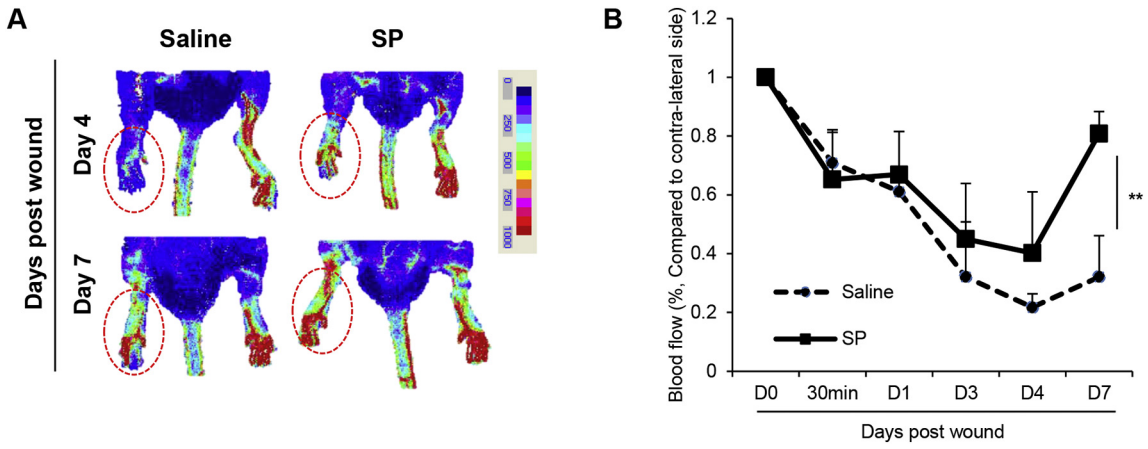

Fig. 3. SP restores blood flow in ischemic zone.
(A) Laser Doppler scanning of the blood flow over the hind-limbs was conducted on day 0, 1, 3, 4, and 7 after the wound was created. (B) The ratio of the ischemic/normal blood flow was calculated. Data are shown as mean values ± standard deviations. n = 8 per group. Scale bar: 200 μm. (*p < 0.05, **p < 0.01, and ***p < 0.001).

3.2. SP reinforces the formation of an elaborate vascular network in the ischemic wound

As shown in Fig. 3, SP treatment could restore blood flow, which might surmount the hypoxic environment. This effect might be inferred to be occurred by neovascularization. In order to analyze vascularization quantitatively, total vasculature and the coverage of pericytes were assessed in the skin and muscle layer within the wound site, respectively.

Fig. 4 reveals that SP treatment could cause large vessel formation with high perfusion (Fig. 4A, white dotted line/arrow head) at the injured site and elevated levels of angiogenic factors including VEGF and TGF-β in the serum (Fig. 4B, saline: 45.4 ± 7.18 pg/ml, SP: 83.21 ± 9.41, saline vs SP < 0.001; Fig. 4C, saline: 1514.2 ± 268.5 pg/ml, SP: 2348.2 ± 78, saline vs SP < 0.001). As VEGF and TGF-β are essential for vascularization, a higher level of VEGF or TGF-β in the SP-treated group may be closely associated with the restoration of vascular network in the ischemic zone.

Immunohistochemical staining proved that in the skin layer, the number of $CD31^+$ vessels in the SP-injected mice was approximately three-fold greater than that in the saline-treated mice; furthermore, α-SMA^+ perivascular cell-covered vessels were detected much more frequently in the SP-injected mice than in the saline-treated mice (Fig. 4D–F and Supplementary Fig. 1). This restoration of tight vasculature with perivascular cells by SP might contribute to the rapid cutaneous healing shown in Figs. 1 and 2.

Ischemic stress in preclinical limb ischemia model was induced by the removal of an artery in the muscle layer. Thus, reconstructing blood vessels in the muscle layer can resolve ischemia, although femoral artery can be rarely regenerated. The quantification of vessel formation in the muscle layer confirmed that SP treatment increased not only the total number of vessels but also α-SMA^+ perivascular cell-covered vessels (Fig. 4G–I).

These results corroborate that SP treatment can induce extensive vascular network formation under hind-limb ischemia.

3.3. SP suppresses ischemic injury-induced inflammatory responses

A cutaneous wound leads to the induction of inflammatory responses. Prolonged inflammation interferes with regeneration, leading to irreversible damage. Moderating the initial inflammation is therefore the first prerequisite for healing [32]. The quantitative analysis for inflammatory indicators at 7 days post wound revealed that SP treatment ameliorated injury-induced inflammation responses by reducing spleen weight and TNF-α levels and increasing IL-10 levels (Fig. 5A–C: TNF-α, saline: 82.2 ± 9.2 SP: 45.8 ± 1.02 pg/ml saline vs SP: p < 0.001; IL-10, saline: 25.03 ± 4.03, SP: 48.23 ± 6.21 pg/ml, saline vs SP: P < 0.001; spleen weight, saline: 7.62 ± 0.42, SP:4.3 ± 0.55, saline vs SP: p < 0.05).

SP has previously been reported to suppress inflammation by modulating M1/M2 monocyte transition at early time points (4.5 h) post injection in several disease conditions, including diabetic ulcer, rheumatoid arthritis, and spinal cord injury [27–30,40]. Thus, it was expected that SP injection would exert immune-suppressive effects on ischemic ulcers at early time points post treatment, leading to accelerated healing. To examine the effect of SP on the initial inflammatory response, TNF-α and IL-10 levels in serum samples were quantified 4.5 h post SP injection (Fig. 5D and E); TNF-α levels decreased and IL-10 levels increased. The main sources of TNF-α and IL-10 are circulating monocytes of the M1 and M2 types, respectively. Therefore, the increase in IL-10 levels and decrease in TNF-α levels suggest the presence of M2 type monocytes. To confirm this, the number of $CD206^+CD11b^+$ monocytes in the blood was determined using FACS. As shown in Fig. 5F, SP treatment elevated the pool of the $CD206^+CD11b^+$ monocytes in circulation, compared with that in the saline-injected group (Fig. 5F and Supplementary Fig. 2).

To determine whether systemic immune modulation by SP affected the macrophage type at the injured site, immunohistochemical staining was carried out for CD206, the marker of M2 macrophages (Fig. 6A). A number of immune cells was detected in the injured site in both groups, but the number of $CD206^+$ immune cells was higher in SP-treated group than in the saline-treated group; this finding was confirmed via quantification of $CD206^+$ cells (Fig. 6B).

This result demonstrated that SP can inhibit ischemia-induced inflammation at the initial stage, the effects of which might affect cutaneous wound repair.

3.4. SP elevates the circulating BMSCs level under ischemic conditions

BMSCs have been shown to improve wound healing by controlling immune responses or by incorporation into the injured tissue. [23] Additionally, BMSCs secrete angiogenic factors such as VEGF as well as immune-suppressive cytokines including PGE2 and IL-10, leading to promotion of neovascularization [32–38]. Thus, the mobilization of BMSCs is expected to have a beneficial effect on ischemic ulcers by reducing highly activated immune responses and inducing angiogenic factors. Hong et al. found that SP can mobilize BMSCs into the circulation to engage them in tissue repair, accompanied by angiogenesis

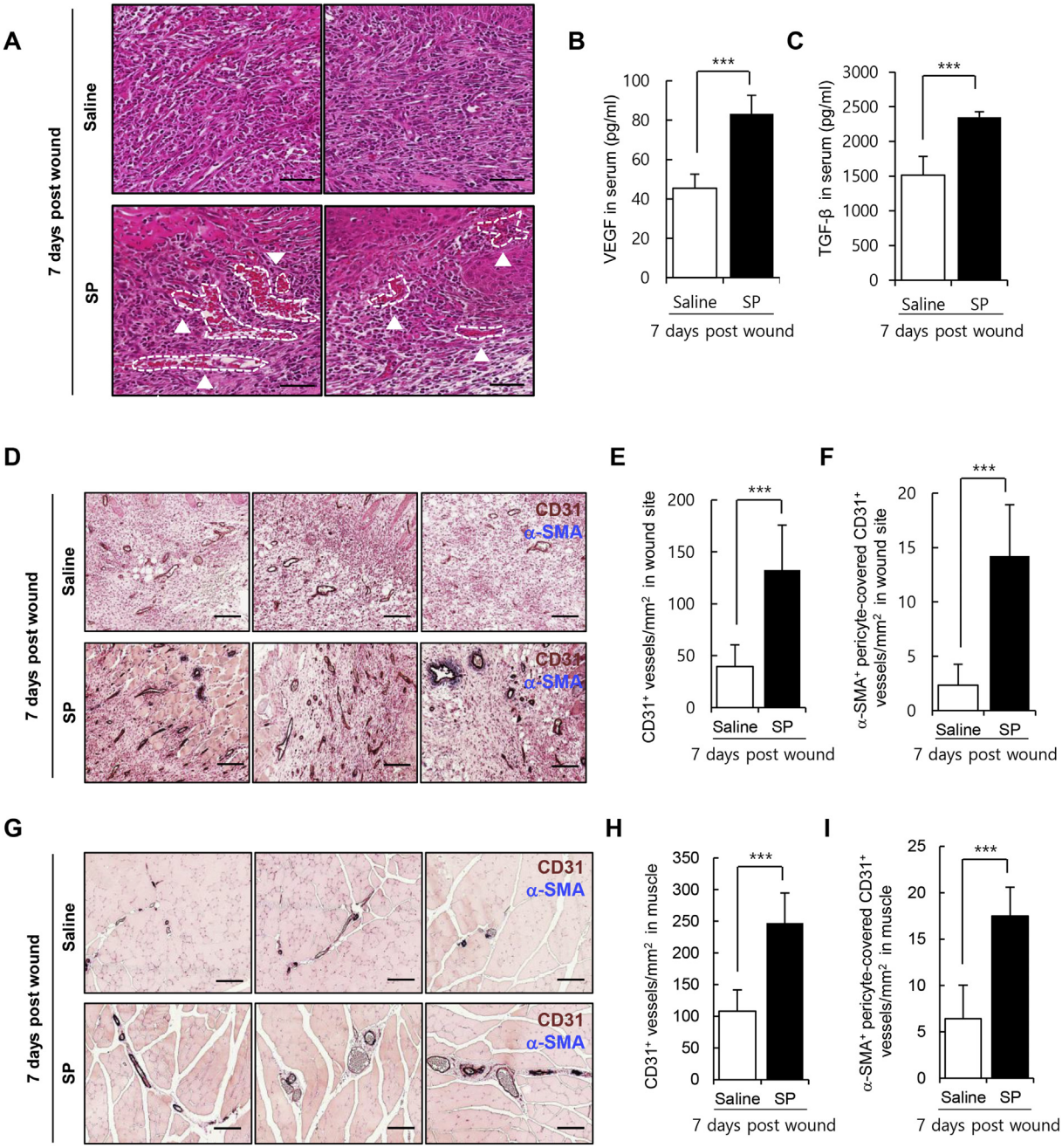

Fig. 4. SP reinforces the formation of an elaborate vascular network in the ischemic wound zone.
(A) Wounds were stained with hematoxylin and eosin on day 7 after they were created. The white dotted circles indicate vessels containing red blood cells. (B–C) The VEGF and TGF-β level in serum samples was quantified using an ELISA. (D) Immunohistochemical staining of CD31 and α-SMA was performed to analyze the structure of the vessels at the dermal layer of the wound sites. (E, F) A quantitative analysis of the number of $CD31^+$ vessels and $\alpha\text{-SMA}^+$ cell-covered $CD31^+$ vessels within the dermal layer of the wound sites. (G) Immunohistochemical staining of CD31 and α-SMA was performed to analyze the formation of vessels at the muscle layer of the wound sites. (H, I) A quantitative analysis of the total number of $CD31^+$ vessels and $\alpha\text{-SMA}^+$ cell-covered $CD31^+$ vessels within the muscle layer of the wound sites. Data are shown as mean values ± standard deviations. n = 8 per group. Scale bar: 50 μm. (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and VEGF in vivo [23–25].

In order to determine the effect of SP on stem cell mobilization under ischemic injury, circulating stem cells were checked using FACS. As expected, analysis of the circulating $CD29^+CD45^-CD105^+$ BMSCs level found that the SP injection augmented the level of $CD29^+CD45^-CD105^+$ BMSCs in circulation under ischemic conditions (Fig. 7 and Supplementary Fig. 3).

Therefore, SP is capable of mobilizing stem cell into the circulation and SP-induced BMSCs may play a beneficial role during ischemic wound healing systemically or locally.

4. Discussion

The fundamental treatment for peripheral artery diseases is thrombolytic or interventional therapy in the clinic. However, these treatments lead to a high risk of hemorrhage and even elevate the risk of mortality; thus, they may not be appropriate for all patients [36]. In addition, although many clinical trials were designed to resolve

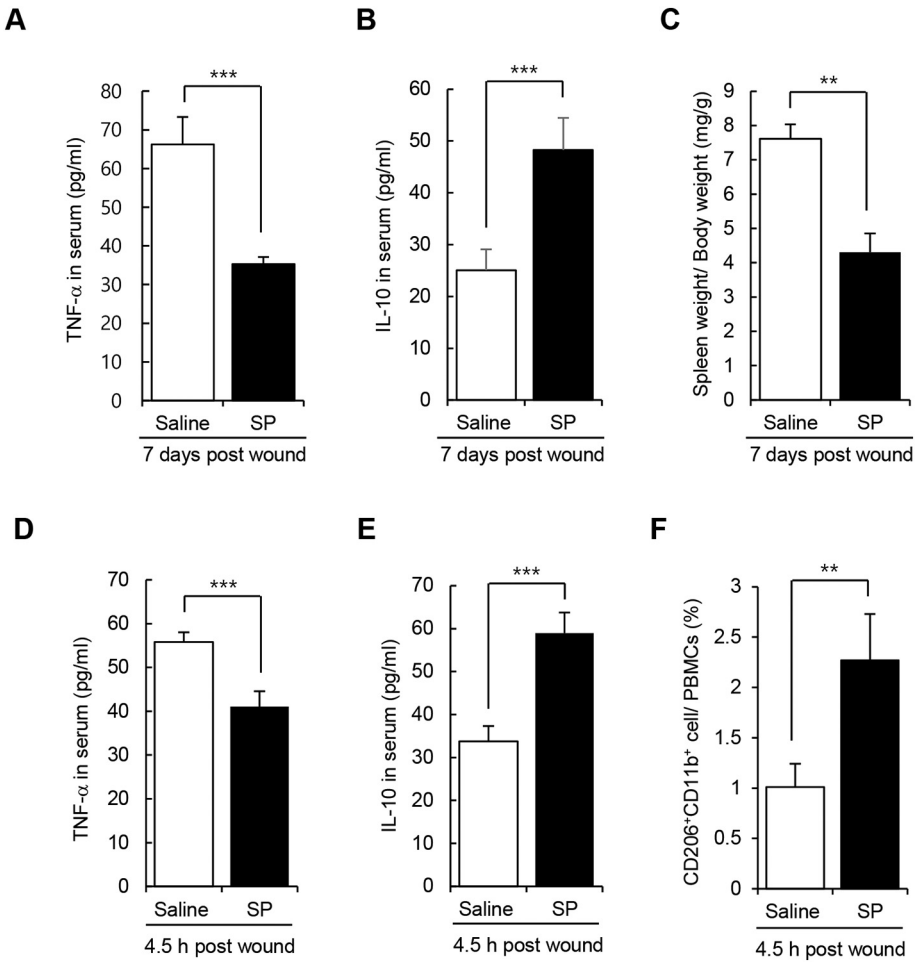

Fig. 5. SP ameliorates ischemic wound-induced inflammation responses at an early time point.
(A–B) At 7 days after SP injection, blood was collected and TNF-α and IL-10 levels in serum samples were quantified using an ELISA (C) Spleen weight was evaluated. (D–E) At 4.5 h after SP injection, blood was collected and TNF-α and IL-10 levels in serum samples were quantified using an ELISA. (F) The M2 monocytes of PBMCs were determined using FACS. Data are shown as mean values ± standard deviations. n = 8 per group. (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

ischemic diseases, they failed to see improvements. The key targets in the treatment of ischemic ulcers are to restore the bloodstream and promote wound healing.

SP can promote cell proliferation, and systemically control immune responses [23,25,28–30]. Injecting SP into wounds of diabetes patients has a therapeutic effect because it modulates inflammation and restores the vasculature [27]. Interestingly, SP itself could induce monocyte polarization into M2 type, immune suppressive form, and this was mediated via PI3K/Akt signaling pathway, accompanied by IL-10 generation [39].

Additionally, SP was able to promote stem cell mobilization by elevating stem cell pools in the bone marrow in diabetes and in those with irradiation-induced damages [23,27,30]. Mobilized BMSCs participate in tissue repair and angiogenesis with VEGF and TGF-β secretion [16,27,29,32].

The purpose of this study was to elucidate whether systemically administered SP could promote the repair of ischemic wounds in mice by restoring blood perfusion and suppressing inflammation. Considering the functions of SP reported, systemically administered SP was likely to have a therapeutic effect on ischemic ulcers by increasing M2 monocyte and MSCs in the circulation.

The results of this study showed that SP is capable of accelerating the repair of ischemic wounds by promoting cutaneous wound closure in mice under ischemic conditions. Compared with mice in the saline-treated group, those in the SP-treated group had complete epithelium coverage and mature granulation tissue.

Cutaneous wound healing includes inflammation, proliferation, and remodeling stages. The rapid closure of a wound by SP may be due to the alleviated inflammation. Evaluation of the effect of SP on inflammation revealed that it inhibited the enlargement of the spleen and modulated the profiles of the cytokines IL-10 and TNF-α in the circulation. The SP-induced IL-10 elevation indicates an increased level of M2 monocytes in the blood, which was confirmed by FACS. Moreover, SP-induced immune suppression was corroborated locally by the increase in the M2 macrophage pool at the injured site.

SP-mediated wound healing was also accompanied by improved blood perfusion and elevated serum VEGF and TGF-β levels. Our histological data also revealed that the vasculature of mice treated with SP was covered with more α-SMA$^+$ perivascular cells than that of mice treated with saline. This difference in vascular structure reflects the distinction of the function of the vascular network due to the formation of a tight vasculature. This SP-induced vascular remodeling implies that vascular activity in the ischemic zone was improved upon SP injection.

SP treatment was also seen to facilitate reparative stem cell mobilization into the circulation under ischemic conditions. Although we did not prove the direct incorporation of SP-mobilized MSCs into the vasculature at the wound site in this study, SP-mediated stem cell mobilization might be beneficial to ischemic wound repair by participating directly in tissue repair through differentiation or by providing paracrine factors.

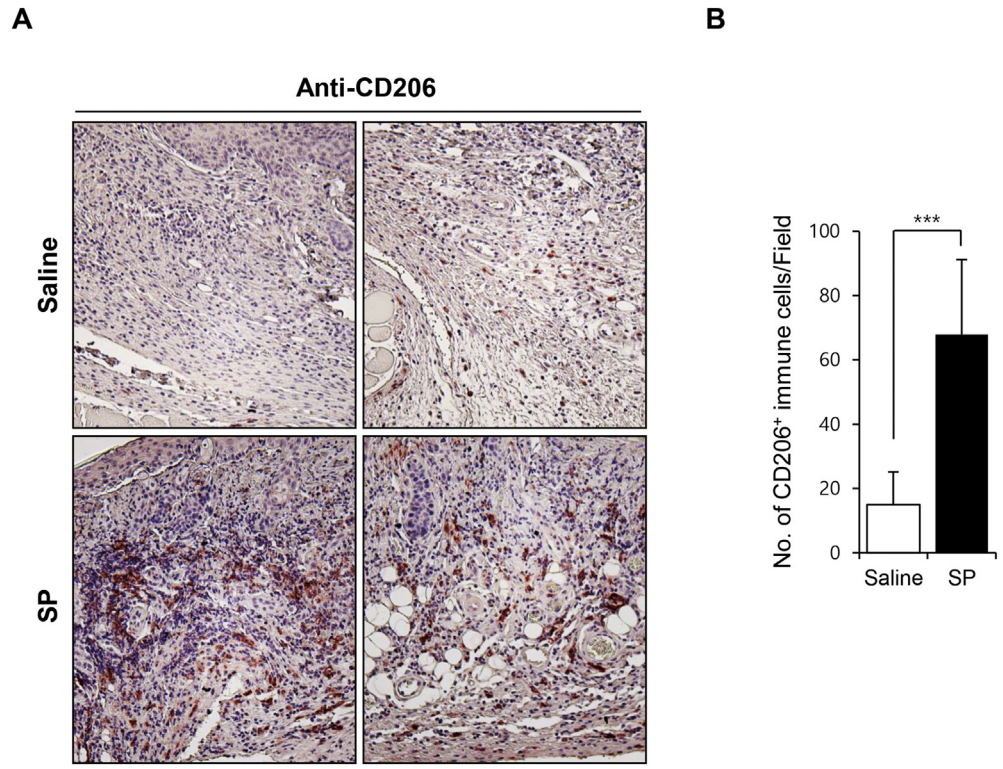

Fig. 6. SP suppresses infiltration of M2 monocytes into the wound.
(A) Immunohistochemical staining for CD206 was carried out to determine infiltrated M2 monocytes. (B) Quantification of $CD206^+$ cells in the injured site was carried out by counting $CD206^+$ cells per field. (*p < 0.05, **p < 0.01, and ***p < 0.001).

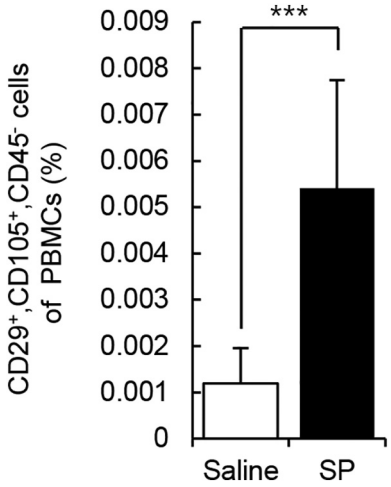

Fig. 7. SP promotes BMSC mobilization in ischemic wound conditions.
At 24 h after SP injection, blood was collected and PBMCs were isolated using a Ficoll gradient. The percentage of $CD45^-CD29^+CD105^+$ MSCs in PBMCs was examined with FACS. Data are shown as mean values ± standard deviations. n = 10 per group (*p < 0.05, **p < 0.01, and ***p < 0.001).

5. Conclusions

Altogether, the results of this study revealed that systemically administered SP accelerates the recovery of cutaneous wounds, induces the recovery of blood perfusion, and suppresses inflammation, leading to the promotion of artery removal-induced ischemic ulcer healing.

Preclinical safety studies on SP have found that intravenous SP administered at the dose that was used in this experiment is not toxic [41]. Accordingly, SP is expected to be a new therapeutic candidate for patients with PAD, including those with ischemic ulcers.

Abbreviations

α-SMA	alpha-smooth muscle actin
BMSCs	bone marrow mesenchymal stem cells
ELISA	enzyme-linked immunosorbent assay
IL-10	interleukin-10
PAD	peripheral artery disease
PBMCs	peripheral blood mononuclear cells
RT	room temperature
SP	substance P
TNF-α	tumor necrosis factor-alpha
VEGF	vascular endothelial growth factor
VSMC	vascular smooth muscle cell

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Conflict of interest statement

The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.04.015.

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